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TITLE: DIAGNOSTIC TEST FOR GLUCOSE

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Ullited States Patent Office 317919988 3,791,988 DIAGNOSITC TEST FOR GLUCOSE Dieter Josef, Prafteln, and Alfred Lampart, Magden, Switzerland, and Pirmin Schwartz, Weil am Rhine, Ger- .5 many, assignors to Hoffmann-La Roche Inc., Nutley, N.J. No Drawing. Filed Mar. 23, 1972, Ser. No. 237,528 Int. Cl. GOIN 33116 U.S. Cl. 252-408 13 Claims 10 ABSTRACT OF THE DISCLOSURE An improved diagnostic composition for the quantitative determination of glucose in biological fluids comprising glucose oxidase, a phenazin and/or phenoxazin-de- 1,5 rivative and a tetrazolium salt as well as the incorporation thereof upon a bibulous carrier are described. DETAILED DESCRIPTTON OF THE INVENTTON 20 The present invention relates to new and improved diagnostic compositions which are useful in both the quali- tative and quantitative determination of glucose in fluids, particularly body fluids such as blood, serum, plasma, urine and the like. 25 The quantitative determination of glucose in body fluids is of great importance to diabetic individuals who must - have frequent checks on the level of glucose in their body fluids as a means of regulating the sugar intake in their diets. The qualitative determination of glucose in body 30 fluids is of importance in routine testing of patients in doctor@' offices, clinics, institutions, and hospitals as well as in the mass screening of individuals for the presence of the disease. The ideal diagnostic composition for the detection of 3,5 glucose in fluids must be simple so as not to require a high degree of techiiical skill on the part of the technician administering the test, sufficiently rapid to be utilized in large scale qualitative screening determinations and be sensitive and accurate enough to be useful to the clinician. 40 Additionally, such a composition must be sufficiently stable to meet all situations such as prolonged storage and the advance preparation of solutions for large scale screening operations. The present invention pertains to such a composition. 45 In recent years the methods of determining glucose concentration in fluids have been primarily based on the enzymatic oxidation of glucose with glucose oxidase and oxygen with subsequent detection and measurenient of the resulting peroxide or gluconic acid oxidation products 50 by means of a color reaction' Such methods suffer from the disadvantage of having fluctuations in results due to variations in the oxygen content of different samples. Fur- ther, with such methods it can never be guaranteed that oxidation process proceeds quantitatively. 55 One attempt to overcome the above described disad- vantage is the addition of an endogeneous oxygen donor, e.g., hydrogen peroxide or the like to the oxidation solu- tion, thereby limiting the measurement of glucose to the relatively insensitive gluconic acid determination. The dis- 60 advantage of this approach is the comparative insensitivity of the gluconic acid determination. The method and

composition of the present invention affords a means whereby the enzymatic oxidation may be utilized to quantitatively determine glucose content with- 65 out the disadvantages of prior art processes. Additionally, the method of the invention has the advantage over the prior art of being equally accurate under aerobic and anaerobic conditions and by not being hampered by false positive results due to the presence of ascorbic acid. 70 In the method of the present invention, the phenazin- and/or the phenoxazin derivative enters into the enzy- Patented Feb. 12, 1974 2 matic oxidation reaction as a hydrogen acceptor. The reduced form of the phenazin- and/or the phenoxazin-derivative (phen,ed) thus formed reacts rapidly in a nonenzymatic reaction with the tetrazolium salt to regenerate the original phenazin and/or phenoxazin-derivative (phen ,.) apd form a forinazan compound which is stable and possesses an intense color. The amount of glucose present is then measured by colorimetrically reading the color of the formazan compound. The reaction may be represented as follows: glucose + phen., @ gluconic acid + phen,.d phen,.d+ tetrazoliura @ phen@. + formazan Phenazin-derivatives which can be present in the agent provided by the present invention include phenazinium salts in particular methc)- and etho-compounds. Phenazinium methosulfate is particularly preferred as it is commercially available. Phenoxazin-derivatives include phenoxazinium salts in particular 7-dimethylamino - 1,2 - benzophenoxazinium chloride (meldola blue) or 7-dimethyl-amino-2'-hydroxy1,2-benzophenoxazinium chloride. The selection of a particular tetrazolium salt is not critical to the present invention. Examples of suitable conipounds include thiazolyl blue [2,5-diphenyl-3-(4,5-dimethyl-2-thiazolyl) mono- tetrazolium bromide], nitrotetrazolium blue chloride [3,3'-dianisyl- 4,4'-bis[2(4-nitrophenyl)-5-phenyll-tetrazolium chloride], iodonitrotetrazolium chloride [2-(p-iodophenyl)-3-(pnitrophenyl)- 5-phenyl-tetrazolium chloride], neotetrazolium chloride- [2,2',5,5'- tetraphenyl-3-(4,4'-biphenylene)-ditetrazolium chloride], tetranitrotetrazolium blue [2,2',5,5'-tetranitrophenyl-3(3,3'- dimethoxy-4,4'-biphenylene) -ditetrazolium chloride], triphenyltetrazolium chloride, tetrazolium violet [ 2,5diphenyl-3-(a- naphthyl)-tetrazolium chloridel and tetrazolium blue [3,3'-dianisyl- 4,4'-bis-(3,5-diphenyl) tetrazolium chloride]. Preferred among these are thiazolyl blue, nitrotetrazolium blue chloride and iodonitroletrazolium chloride. The diagnostic compositions of the present invention can be provided in a solid form,, e.-g., tablets or powder or,-preferably, as a solution in a suitable solvent. Such solutions may be utilized per se or impregnated onto a bibulous carrier such as filter paper, cardboard and the like. Wherein solutions of the diagnostic compositions of the invention are contemplated, aqueous solutions are preferred. It is expedient, however, to add an organic solvent, e.g., a lower alkanol such as ethanol or a solubilizing agent, e.g., polyethylene glycol, polyoxysorbitanic acid ester or the like to hold in solution the water-insoluble formazans which are formed in the determination reaction. A preferred polyethylene glycol is polye col 1500 -and a preferred polyoxysorbitanic acid ester is that marketed by Atlas Chemical Industries, Inc., Wilmington, Del. under th6 trademark Tween 8 1. This substance chemically is polyoxyethylene(5)sorbitan monooleate. In place of a solvent, a suspending agent such as gelatin or an alginate may be utilized to form a suspension of the formazans as they are produced. The compositions of the present invention are prepared by simply mixin.- together the phenazin and/or phenoxazin- derivative, the tetrazolium salt and glucose oxidase. While the relative concentrations of the components is

3;7912988 3 not particularly critical, it is preferred to have from about 0.1 to about 2.5 millimoles of phenazin and/or phenox- azin-derivative and from about 1.0 to about 10.0 milli- moles of tetrazolium sait present for each 0.5 to about 5.0 mg. of glucose oxidase present. 5 It is preferred in the practice of the present invention to have the pH of the fluid to be tested for glucose content at between about pH 4 and 8, preferably between pH 5.5 and pH 7.5 when the diagnostic composition of the in- vention is admixed therewith. It is therefore preferred to 10 add a suitabie buffer to the diagnostic compositions of the invention. Preferred among the buffering agents common to the art is a phosphate buffer system. Wberein the diag- nostic compositions of the invention are in the form of a solution, said

solution is preferably from about 0.1 to 15 about 0.4 molar with respect to the buffer. Where the compositions of the invention are in a dry form, a suffi- cient amount of buffer is added so that a solution formed therefrom will have the desired concentration of buffer. Altemately, the buffer may be added as a solution to the 20 solution of diagnostic composition before conducting the diagnostic determination. The selection of the particular tetrazolium compound in the practice of the present invention may be determined by the type of test to be conducted. For example, a 25 tetrazolium compound producing a formazan which absorbs light in the spectral range sensitive to the eye can be utilized for qualitative or s emi-quantitative glucose detemiinations such as by the use of bibulous strips im-pregnated with reagent. Where quantitative detenninations 3( are to be carried out utilizing a photometer, the selection of a tetrazolium compound should be made so that the light absorbance of the formazan produced therefrom in the reaction is maximal at the wavelength capacity of the particular apparatus being utilized. The following table il- 35 lustral@s the wavelength absorption maximum of the for-mazan produced from preferred tetrazolium compounds in accordance with the invention. TABLE 40 Wavelength of the absorption ma3dmurn of the formazan in CiDlor of polyethylene the form- TetrazoUum component glycol -azan - 45 Thiazolyl blue ---------- 570 iim ---------- Violet. Tetranitrotetrazolium blue ---------- 530 nm ---------- Blue-red. Nitrotetrazolium blue chloride ---------- 580 nm ---------- Blue. lodonitrotetrazolium chloride ---------- 456 mn ---------- Do. NeotetrazoUun2 chloride ---------- 560 nm- ---------- Red. Triphonylteti-4zolium chloride ---------- 588 nm ---------- Red. Tetrazolium violet ---------- 510 nm ---------- violet. 4 EY-AMPLE 2 The procedure of Example I was repeated utilizing a buffer solution containing in each 100 ml. 1.0 g. of gelatin. As the formazan compound formed in the reaction was held in suspension treatment with ethanol was not necessary. The photometer reading was taken directly after the two minute incubation period. Example 3 The procedure of Example 2 was repeated replacing the gelatin with an equal amount of polyoxysorbitanic acid ester (Tween 81, Atlas Powder Co.). Example 4 The following solution was prepared and impregnated on strips of bibulous material, such as filter paper. The paper was soaked in the solution and dried with the exclusion of light. The thus-formed srips were utilized to s emi-quantitatively determine the glucose content of a sample by immersion therein. Ingredient: Amount Glucose oxidase ---------- mg--. 500 Phenazinium methosulfate ---------- mg-- 10 Thiazolyl blue ---------- mg-- 500 0.5 M phosphate buffer pH 6.6 ---------- ml-- 50 Distilled water ---------- ml-- 40 Example 5 The following solution was prepared for the quantitative determination of glucose. Component: Amount, ml. Glucose oxidase (20 mg./ml.) ---------- 0.05 Meldola blue (2 mg./ml.) -- ---------- 0.05 lodonitrotetrazolium chloride (2.5 mg./ml.) ---- 0.40 Buffer (0.2 M potassium phosphate pH 7.0 1% polyethylenglycol 1500) ---------- 1.50 To this solution is added I ml. of a sample solution. The content of glucose is determined by measuring in a photometer the extinction difference in comparison with a blank test in the wavelength range of about 492 nm. What is claimed is: 1. A diagnostic composition for the detection of glucose in fluids consisting essentially of glucose oxidase, a tetrazolium salt and a substance selected from the group consisting of a phenazinium salt, a phenoxazinium salt and mixtures thereof and one or more non-reactive ad- Tetrazolium blue ---------- 570 nm ---------- Blue. 60 juvant materials selected from the group consisting of buffers, solubilizing agents and suspending agents. The following examples serve to further illustrate the 2. The composition of claim I wberein said buffer is invention. suitable for maintaining the pH of said fluids between EXAMPLE 1 .55 pH 4 and 8. 3. The com posi tion of clai m 1 whe rein said phe nazi - The following solution was prepared for the quantitative nium salt is selected from the group consisting of phenazi- determination of glucose: nium metho- and phenazinium etho- salts. 4. A test indi cato r for dete ctin g gluc ose in fluid s con- Concentra- 60 sisting essentially of a bibulous carrier impregnated with tion/ml. Amou nt the composition of claim 1. of solution of solution Component tboreof used 5. A test indicator for detecting glucose in fluids con- ]Buffer-potassium gho3hate pH 7.0 --------- 0.3 M ------- ILO nil. sisting essentially of a

bibulous carrier impregnated with Phenazinium met os ate ---------- 2mg ---- ---- 0.05:ml. the composition of claim I wherein said phenazinium salt Thiazolyl blue ---------- 10 mg ------- 0.4 ml. 6,5 is phenazinium methosulfate and said tetrazolium salt is Glucose oxidase ------ ---------- ----- 20 ing ------- 0.05 ml. thiazolyl blue. 6. A test indi cato r for dete ctin g gluc ose in fluid s con- The above solution was combined with I ml. of the fluid to be tested and allowed to stand at room temperature for about 2 minutes. The mixture was then treated with an 70 equal volume of ethanol and measured in a photometer. Similar readings were taken utilizing standard solutions containing known amounts of glucose. The glucose corltent of the sample was then determined by reference to a calibration curve. 7,5 sisting essentially of a bibulous carrier impregnated with the composition of claim I wherein said phenazinium salt is 7-dimethyl amino-1,2@benzophenoxazinium chloride and said tetrazolium salt is iodon itrotetrazolium chloride. 7. A diagnostic composition for the detection of glucose in fltiids consisting essentially of a solution of the composition of cM!m I in a solvent consisting essentially of water and a lower alkanol.

5 S. A diagnostic composition for the detection of glucose in fluids consisting essentially of an aqueous solution of the composition of claim 1 wherein said solubilizing agent is selected from the group consisting of polyethylene glycol 1500 and polyoxyethylene(5)sorbitan monooleate. 9. A diagnostic composition for the detection of glucose in :fluids consisting essentially of an aqueous solution of the composition of claim 1 wherein said suspending agent is selected from the group consisting of gelatin and an alginate. 10. The composition of claim 2 wherein said phenoxazinium salt is selected from the group consisting of 7-dimethylamino-1,2-benzophenoxazinium chloride and 7dimethylamino-1,2-benzophenoxazinium chloride. 11. A test indicator for detecting glucose in fluids consisting essentially of a bibulous carrier impregnated with the composition of claim 2. 12. A diagnostic composition in accordance with claim 3,791,988 6 2 wherein said tetrazolium salt is selected from the group consisting of thiazolyl blue, nitrotetrazolium blue, iodonitrotetrazolium chloride, neotetrazolium, triphenyltetrazolium chloride, tetrazolium velvet, tetrazolium blue and tetranitrotetrazolium blue. 1'3. The composition of claim 3 wherein said phenazinium metho-salt is phenazinium methosulfate. References Cited 10 UNITED STATES PATENTS 3,123,443 3/1964 Smeby ---- ------- 252-408 X 3,233,974 2/1966 Bradley ---------- 252-408 X 1, MAYER Vv'EINBLATT, PrimaryExaminer U.S. Cl. X.R. 23-230 B, 253 TP; 424-7

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